

THE ALLERGICALLY ACTIVE SUBSTANCE IN RAGWEED POLLEN. A CHEMICAL AND BIOLOGICAL STUDY*

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INTRODUCTION

The object of this work has been the isolation and identification of the fraction or fractions of pollen which were specifically active in clinical cases of hay fever. The fractions giving an immediate urticarial type of cutaneous reaction have been studied. No attempt was made to identify inactive material. The importance of a close relationship between chemical work and biological tests was evident.

In the preliminary report, two substances were described. Substance A, containing a large amount of carbohydrate, gave positive skin reactions in 90 per cent of a series of ragweed-sensitive cases and in 10 per cent it was inactive. Substance B, of protein nature and free from Substance A, gave reactions in all of the ragweed-sensitive cases tested.

Subsequent work here reported will show that Substance A, which could not be prepared free from nitrogen, was a mixture of small amounts of Substance B with carbohydrate material, since tests have shown that the active material in the so called Substance A was biologically identical with Substance B. Substance B has been isolated in a comparatively pure state and corresponds on chemical analysis and by its chemical reactions to a plant albumin.

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HISTORICAL

Heyl (1), in a chemical analysis of ragweed pollen, isolated twenty-seven substances, but due to insufficient clinical tests with the products the results were disappointing in regard to allergic activity. Fatty acids, lecithin, resins, sucrose, glucose, nitrogenous bases, and phytosterols were isolated. A fraction composed of 75 per cent albumin and 25 per cent proteose had anaphylactogenic properties. Koessler (2) studied the nitrogen distribution in ragweed extracts and Gottlieb and Oppenheimer (3) prepared active material by alcoholic precipitation from saline extracts. Paton (4) reported the presence of eight enzymes in pollen. Bauman, Chudnoff, and Mackenzie (5) reported the presence of active material in a fraction that they considered globulin. Caulfield, Cohen, and Eadie (6) described an albumin-proteose fraction giving positive skin reactions. Coca and Grove (7) and Black and Moore (8) from a study of extracts concluded that the active substance of pollen was non-protein. Moore and Moore (9) have made a study of the nitrogen distribution in extracts of timothy, orchard grass, and ragweed pollen and Moore, Cromwell, and Moore (10) have described fractions of varying activity. Bernton, Jones, and Csonka (11) concluded from a preliminary study of protein fractions from timothy and ragweed pollen that the activity was not restricted to a single fraction.

EXPERIMENTAL

The pollen of the giant ragweed (*Ambrosia trifida*) has been used in this work except where otherwise specified. Preliminary work with timothy, low ragweed, and plantain pollen has indicated active material of a similar nature. Tests for activity have been made with solutions in bicarbonate-extracting fluid (12), buffered saline (13), or normal salt, that were in some measure comparable to the routine ragweed extracts. Whenever insoluble, the material was tested by the scratch method.

The direct method of testing extracts intradermally on sensitive individuals, while valuable for establishing activity, is not reliable for determining the amount of active substance. With the direct method a certain extract will often give an immediate wheal apparently identical with that produced by the same extract diluted 100 times.

In this study it was frequently necessary to determine the relative amounts of allergen by comparing certain fractions of the pollen with each other or with the routine extract of defatted pollen. Such comparisons may be quite accurately made by the indirect method (passive transfer of Prausnitz and Küstner (14)) as follows: An accurately measured amount (0.05 or 0.1 cc.) of serum

of ragweed-sensitive patients was injected into the skin of non-sensitive individuals. Three such sites were usually made in each arm. 2 or 3 days later measured amounts (0.05 or 0.1 cc.) of the extracts to be tested were injected into sensitized sites. Immediate reactions took place with the result that the sites were partially or completely desensitized. When any site was inactive to its own test extract it was then tested with the other solution which was being compared. Occasionally sites, frequently tested, may become refractory from tissue change or fatigue. In this event a negative reaction is not due to specific desensitization and may be

TABLE I
Comparison of Purified Albumin Extract 991 with Ragweed Extract, by Desensitization

Date of test..	Aug. 11			Aug. 13							
Sites*	0.1 cc. extract	N found per cc.	Result	0.05 cc. extract	N found per cc.	Result	0.05 cc. extract	N found per cc.	Result	Extract	Result
		mg.			mg.			mg.			
1, right	R	0.1	++++	R	0.1	—	991	0.1	—	Horse serum	++++
2, “										“ “	++++
3, left	991	0.1	++++	991	0.1	—	R	0.1	—	“ “	++++
4, “										“ “	++++
Control	R	0.1	—								
“	991	0.1	—								

* Injections in these sites were made on August 9.

misleading. To guard against this, sera were selected from cases sensitive to ragweed and one or more unrelated antigens, for example, horse serum. After ragweed tests were completed in any study and especially in cases where negative results were obtained, a test was made with horse serum and, if positive, refractoriness was ruled out. The negative response to ragweed extract was due to specific desensitization. In instances where sera used did not contain antibody to unrelated antigen the method was devised of supplying this by the addition of serum containing an antibody different from the one used for sensitizing sites for tests.

An example to be referred to later in the text is here cited (Table

I). Serum 30 was obtained from a patient suffering from asthma and hay fever and sensitive to ragweed and horse serum. On August 9, 0.1 cc. of this serum was injected intradermally into each of four sites, two on the right arm and two on the left arm of a non-sensitive individual. Extract 991 (purified albumin) was tested against a routine ragweed extract, Extract R, of defatted pollen.

This experiment, which is one of many, shows that the routine ragweed, Extract R, and the purified albumin of ragweed, Extract 991, were active in Sites 1 and 3 respectively. The second test with these substances in the same site 2 days later was negative; that is, the sites were desensitized. When Extract 991 was then put in Site 1 there was no response and when Extract R was put in Site 3 there was no reaction. This shows that the routine ragweed extract contained no active substance other than the albumin in Extract 991 for if another allergen were present a reaction would have resulted. Refractoriness was excluded by the subsequent reaction to horse serum. The desensitization was considered specific. This showed that the purified albumin contained the active substance present in the routine extract of ragweed pollen.

The extracts from whole pollen were made by removing the fatty material with ether or petroleum ether before aqueous extraction was carried out. This fatty oil when removed from dry pollen under anhydrous conditions was free from nitrogen and did not give immediate specific skin reactions. It was slightly water-soluble. In some cases on scratch test it produced a dermatitis as reported by Milford (15) and Bengtssen (16).

Sections of whole pollen grains stained with fat stains showed that this oily material was contained in the outer burr or shell of the pollen, acting more or less to inhibit water extraction.

1. Preliminary Work

A. Influence of Heat upon Activity of Pollen and Extracts

No allergically active volatile product could be isolated by a destructive distillation of whole ragweed pollen. A steam distillation gave a distillate with a pungent pollen odor, containing some ether-soluble fatty material with no skin activity. Activity remained in the distillation flask with the pollen residue.

The activity of dry whole ragweed pollen or petroleum ether-

defatted pollen was not measurably reduced by heating at 100–120° for 2 hours. An additional heating for 15 minutes at 200° resulted in browning the pollen with loss of activity.

Gay (17) made a study of the destructive action of heat under different conditions of dilution and pH concentration.

Tests, of passively sensitized sites, by the indirect method described on page 570 showed that heating Extract 901 for 1 hour in a boiling water bath had decidedly reduced its desensitizing capacity in comparison with the unheated preparation, Extract 901.

B. Chemical Analysis of Giant Ragweed Pollen

	<i>per cent</i>
Total volatile matter (102°).....	5.99
Vacuum drying loss.....	4.00
Ash.....	5.94
Nitrogen (dry whole pollen).....	4.11
“ (“ defatted pollen).....	4.63

Qualitative tests showed the presence of phosphorus but no sulfur. The ash was largely Ca and PO₄.

Material Removed by Extraction of Dry Whole Pollen with Anhydrous Solvents (Soxhlet)

	<i>per cent</i>
Petroleum ether, b.p. 30–50°.....	10.9
Benzene, b.p. 80°.....	11.8
Chloroform.....	15.2
Ethyl ether.....	11.3
Absolute alcohol.....	32.7

Petroleum ether, dry ethyl ether, and benzene extracts were found nitrogen-free by sodium fusion. Chloroform and 95 per cent alcohol extracts containing nitrogen and phosphorus showed faint activity with some loss of activity in the residues. A 95 per cent alcohol extract of dry pollen residue following water extraction was inactive.

In all subsequent work petroleum ether-defatted ragweed pollen was used.

Material Removed by Successive Extraction

3 gm. of ragweed pollen were extracted at room temperature with successive 100 cc. volumes of absolute alcohol, 95 per cent alcohol,

and 70 per cent alcohol and water. Nitrogen was determined on each extract as well as on the dry residue and the amount of material removed in alcoholic extracts was calculated from the weight of the dry residue. On evaporation of 10 cc. of the clear water extract to dryness at 100°, 0.0093 gm. of material resulted. This material contained 17 per cent ash, mainly calcium phosphate, and 8.6 per cent nitrogen calculated from the nitrogen in 10 cc. of the extract. The results are given in Table II.

The water extract contained the bulk of activity and therefore its nitrogen included the active nitrogen. Since 3 gm. of pollen in 100 cc. of water gave extracts of about 0.3 mg. of nitrogen per cc., but one-third of the water-extracted nitrogen was related to

TABLE II
Material Removed from Ragweed Pollen by Successive Extraction

	Material extracted	N found per cc.	N removed	Activity
	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>	
Absolute alcohol.....	12.2	0.14	0.45	—
95 per cent “.....	8.5	0.10	0.36	—
70 “ “ “.....	14.1	0.18	0.58	+
Water.....	3.0	0.08	0.25	++++
Residue.....			3.32	—
Total.....	37.8	0.50	4.96*	

* The defatted pollen contained 4.63 per cent N.

the activity. The 70 per cent alcohol contained some active material. Subsequent work showed the importance of these data.

Determination of pH of Pollen Extracts

A colorimetric determination of the pH of pollen extracts showed that distilled water extracts of ragweed and timothy pollen had a pH of 5.3 to 5.5. Bicarbonate-extracting fluid extracts had a pH of 7.6. A water extract of ragweed pollen which had been first extracted with absolute alcohol had a pH of 6.2. The absolute alcohol extract after evaporation and after being taken up in water had a pH of 4.8.

It seemed probable that the bicarbonate-extracting fluid favored the stability of routine extracts by giving slightly alkaline solu-

tions. Since the isoelectric point of most plant proteins (18) lies in the range of pH 4 to 5, the most favorable range for their precipitation, slight alkalinity favors the stability of these colloidal solutions.

C. Nitrogen Content and Activity of Water and Bicarbonate Extracts

18 hour water extracts of ragweed pollen prepared from 3 gm. of pollen per 100 cc. contained 0.3 mg. of nitrogen per cc., while a similar bicarbonate extract contained 0.4 mg. of nitrogen per cc. Skin tests showed no detectable difference in activity; hence the active material was as soluble in water as in alkaline medium.

TABLE III

Water Extracts of Ragweed Pollen and Pollen after Additional Cold Absolute and 95 Per Cent Alcohol Extraction

Prepared from 3 gm. per 100 cc.

	Pollen	Pollen after absolute and 95 per cent C ₂ H ₅ OH extraction
Material removed by water, <i>per cent.</i>	29.6	21.6
N per cc., <i>mg.</i>	0.32	0.26
Free reducing substances as glucose, <i>per cent.</i> ...	1.0	0.0
Total reducing substances after hydrolysis, <i>per cent.</i>	5.2	5.5
Activity.....	++++	++++

A 3 hour bicarbonate extraction of ragweed pollen was as complete as a 6, 18, or 72 hour extraction, showing the ready solubility of active material. Extracts of long duration were objectionable due to yielding turbid extracts giving precipitation on standing.

No difference in activity could be determined by direct skin tests in extracts made with different amounts of pollen in the same volume of extractive when dilutions were made on a nitrogen or weight basis.

A study of twelve successive water extracts of ragweed pollen showed that a 3 hour water extraction removed practically all the extractable nitrogen and all the reducing substances with the exception of a small constant factor as well as most of the active material.

D. Reducing Substances in Pollen and Pollen Extracts

Active extracts of ragweed pollen reduced Benedict's solution, showing free reducing substances with heavier reduction after acid hydrolysis. The reducing substances before hydrolysis were determined by clearing the extracts with mercuric nitrate solution, neutralizing with sodium bicarbonate, removing the last traces of mercury with hydrogen sulfide, and determining the free reducing substances by the gravimetric method of Allihn. Total reducing substances were determined similarly after hydrolyzing the extracts with 10 per cent sulfuric acid in a boiling water bath for 5 hours. Reducing substances are reported either as weight of Cu_2O per volume or as percentage glucose.

The ragweed pollen contained 9.5 per cent reducing substances after hydrolysis. The pollen residue after water extraction contained 4.5 per cent reducing substances.

Cold absolute and 95 per cent alcohol extracts of the ragweed pollen removed 16 per cent of the pollen. These extracts contained only free reducing substances and some water-soluble nitrogen and were inactive (see Table III).

The water extract following the alcohol extraction described above was active and contained no free reducing substances.

In addition to the nitrogen content of the active water extracts the presence of material of a glucosidic nature, giving reducing substances after hydrolysis, indicated that such material might be responsible for activity.

E. Comparison of Nitrogen Content and Reducing Substances in Distilled Water Extracts of Ragweed and Cattail Pollen

Since extensive clinical tests by one of the authors (Cooke) had failed to show sensitivity to the wind-borne cattail pollen (*Typha angustifolia*), it was of interest to compare it with an allergically active pollen such as ragweed (Table IV).

Cattail pollen extracts were free from the froth characteristic of ragweed extracts. Reducing substances were present entirely in the free state in cattail extract since reduction after hydrolysis was less, indicating some destruction during hydrolysis. In ragweed extracts a larger amount of these reducing substances resulted after hydrolysis, indicating material of the nature of a glucoside and the absence of such material in cattail further suggested this material

might be responsible for activity in ragweed. Furthermore, the cattail pollen extract on the addition of alcohol to 90 per cent concentration gave none of the typical gum precipitated from ragweed extract. This gum gave reduction only after hydrolysis.

Cattail pollen extracts did not precipitate at 50 per cent alcoholic concentration. As will be shown later, the active substance of ragweed pollen extract was precipitated at this point. This probably explains the clinical inactivity of cattail pollen.

F. Precipitation of Active Material from Pollen Extracts by Alcohol or Acetone

Preliminary experiments with distilled water extracts of ragweed, timothy, and plantain pollen showed that when alcohol or acetone was added to produce a final concentration of 90 per cent, the active

TABLE IV
Comparison of Cattail Pollen with Ragweed Pollen

	Ragweed extract, 3 gm. per 100 cc.	Cattail extract, 3 gm. per 100 cc.
Material removed by water, <i>per cent</i>	29.6	26.7
N per cc., <i>mg</i>	0.32	0.29
Free reducing substances as glucose, <i>per cent</i>	1.0	7.2
Total reducing substances after hydrolysis, <i>per cent</i>	5.2	5.3

substance was practically all in the precipitate. The clear, alcoholic or acetone supernatant liquid, after being evaporated and taken up in saline, showed very slight activity by direct skin test. From a water extract, one precipitation by alcohol to 90 per cent concentration gave a precipitate amounting to 7 per cent. This precipitate was not completely soluble in water. The soluble portion was taken up in water and reprecipitated by alcohol at 90 per cent concentration. By repeating this process a water-soluble, active material resulted which contained a high percentage of reducing substances on hydrolysis and was designated Substance A.

In order to have active material for study, 47 gm. of pollen were treated with cold absolute, then 95 per cent alcohol, as described on page 576, in order to remove inert water-soluble nitrogenous material and free reducing substances. The pollen was then

extracted for 4 hours with 350 cc. of distilled water at a temperature of 7°. The clear filtrate at a volume of 350 cc. was treated with 350 cc. of alcohol and within 3 hours a flocculent precipitate settled which when removed by the centrifuge left a clear solution. This material, Precipitate 761, when dry equalled 1.3 per cent of the pollen. To the clear alcoholic solution, alcohol was added to make the final volume 3200 cc. Precipitate 762 settled out after standing for 18 hours and it was collected by the centrifuge, washed with 95 per cent alcohol, dried, and equalled 4.7 per cent of the pollen.

Precipitate 761 was separated into a water-insoluble material, Precipitate 761-a, active on scratch test, which was shown later to be mainly coagulated Substance B and a small amount of carbohydrate material similar to Precipitate 762, Substance A. Precipitate 762 was dissolved in water, filtered, and the solution divided. One portion was precipitated by adding alcohol to 90 per cent, giving Precipitate 762-a; the other portion was similarly precipitated with acetone, giving Precipitate 762-b.

Ragweed pollen was washed in the cold with absolute and 95 per cent alcohol. The pollen residue was extracted with water and the clear filtrate was precipitated by adding an equal amount of alcohol. This precipitate was removed and the clear filtrate was further precipitated by adding alcohol to a final concentration of 90 per cent. This second precipitate was dissolved in water, filtered, and precipitated by the addition of alcohol to 90 per cent concentration for six times, giving Precipitate 856, Substance A (see Table VI).

Since Substance A, as prepared above, was soluble in 70 per cent alcohol, pollen after being washed with cold absolute and 95 per cent alcohol was extracted with 70 per cent alcohol. This filtered extract was precipitated by adding alcohol to 90 per cent concentration. This precipitate was taken up alternately in 70 per cent alcohol and water, and precipitated by the addition of alcohol to 90 per cent concentration for six times giving Precipitate 876.

Solutions containing from 0.2 to 0.3 gm. per 100 cc. of Precipitate 762, 856, or 876 (Substance A) contained nitrogen. The reducing substances after hydrolysis were about equal to routine ragweed extracts. These solutions of precipitates were active in direct skin tests in 90 per cent of a series of sensitive cases. However, 10 per cent of the cases gave reactions to routine ragweed

TABLE V
Comparison by Desensitization of Substance A, Solution of Precipitate 856, and of Substance B, Extract 883
 0.05 cc. of the solution was used in each test and 0.05 cc. of serum was injected into each site.

Date of test...	Nov. 27			Nov. 29			Dec. 1		
	Solution used	N found per cc.	Result	Solution used	N found per cc.	Result	Solution used	N found per cc.	Result
1, right	856	0.01	++	856	0.01	-	883	0.1	-
2, "	856	0.1	+++	856	0.1	-	883	0.1	-
3, "				883	0.1	+++			
4, left	883	0.01	+++	883	0.1	+++	856	0.1	-
5, "	883	0.1	+++	883	0.1	-			
6, "	883	0.01	+++	883	0.01	-	883	0.1	+++

* Injections in these sites were made on November 25.

pollen extracts, but negative reactions to solutions of these precipitates. This at first indicated that there might be two active substances in pollen, one of them this gummy glucosidic material, Substance A.

Attempts to prepare Precipitate 856 or 876 free from nitrogen were unsuccessful. The observation was made that material as first precipitated from the water extract with alcohol was more active in producing sneezing in a sensitive individual than the purified preparations, Precipitate 856 or 876.

In order to determine whether this glucoside-nitrogen-containing fraction (Substance A) was a specific allergen and distinct from the non-glucoside fraction containing nitrogen (Substance B),

TABLE VI
Analysis of Precipitates

Precipitate*	Nitrogen	Phosphorus	Glucose (reducing substances)	Ash†
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
762	4.38	2.58	48.8	
762-a	3.95	3.02	48.8	
762-b	6.53	2.76	48.8	
856	4.46	1.42	57.0	4.9
876	5.70	0.38	60.8	2.4

* All the precipitates on test showed activity.

† The ash contained Ca and PO₄.

desensitization tests were made. The following is a typical example. Results in all cases were identical.

M. G., aged 20 years, not sensitive to ragweed extract, volunteered for the experiment. Three sites were made in each arm with 0.05 cc. of serum in each site. The serum was obtained from a patient sensitive, clinically and by test, to ragweed pollen. The tests were made with solutions of Precipitate 856, the glucoside fraction, and Extract 883, described in detail later, which was free from glucoside. Solutions in each case were made on a nitrogen basis expressed in mg. per cc., 0.05 cc. being used in each test injection. Control tests with a solution of Precipitate 856 and Extract 883 were negative (Table V).

Tests in Sites 1 and 2 showed that Precipitate 856 desensitized sites against its own solution but not against Extract 883 in equiva-

lent solutions. In Sites 5 and 6, Extract 883 desensitized against itself and against Precipitate 856. It is particularly important to note in Site 6 that two injections of Extract 883 (0.01 mg. per cc. N) desensitized against desensitization caused by Precipitate 856 (0.1 mg. per cc. N) but the site was still reactive to Extract 883 (0.1 mg. per cc. N).

The conclusion must be that Precipitate 856 contained a small amount of the same active substance as Extract 883. Since Extract 883 contained no glucoside, this type of substance did not play a part as antigen in ragweed pollen extracts. Our former assumption of two antigens in this pollen was incorrect. There is no active glucoside (Substance A).

The nitrogen contents of Precipitates 856 and 876 (Table VI) were sufficient to explain their activity as due to small amounts of protein material. Further evidence for this conclusion was seen in the reduction of activity on repeated precipitation by alcohol, probably due to denaturation. Complete precipitation of pollen extracts and solutions of Precipitate 856 or 876 with phosphotungstic acid removed all the activity again, indicating the protein nature of the activity. Solutions of Precipitates 856 and 876 gave negative protein reactions, such as biuret, xanthoproteic, and Millon's. The high percentage of carbohydrates obscured these tests, but they gave a Molisch reaction and precipitation with basic lead acetate, phosphotungstic acid, and tannic acid. These solutions formed a froth on shaking, gave a reddish-brown color with concentrated sulfuric acid, and gave reduction only after hydrolysis. Hydrolyzed solutions gave tests for pentoses with aniline acetate paper and phloroglucine. With phenylhydrazine, a typical orange-yellow osazone melting at 160° resulted, indicating arabinose. These solutions were levorotatory before hydrolysis, showing no rotation afterwards, indicating protein material. In general, vegetable proteins are levorotatory. Purified Substance B was levorotatory; which was further evidence of its presence in Substance A.

2. Isolation and Identification of the Pollen Allergen

A. Separation of Substance A from Substance B

Since 70 per cent alcohol removed most of Substance A from the absolute and 95 per cent alcohol-washed pollen, different

strengths of alcohol were tried to see if one could be found which would remove Substance A without removing any great quantity of the active Substance B.

1.5 gm. of the absolute and 95 per cent alcohol-washed ragweed pollen were extracted for 18 hours with 50 cc. of 85 per cent alcohol, Extract 901-a. This extract was collected and the pollen washed with an additional 100 cc. of the same strength alcohol to remove the last traces of soluble material. The pollen residue was dried, weighed, and then extracted with 50 cc. of distilled water (Extract 901). The extracts were analyzed for nitrogen and reducing substances on hydrolysis. The water extract was tested for activity after saline dilution, and the alcoholic extract after evaporation was tested by the scratch test. The procedure was repeated with

TABLE VII
Extractions at Different Alcohol Concentrations

	Extract 901-a (85 per cent alcohol)	Extract 901 (H ₂ O)*	Extract 902-a (80 per cent alcohol)	Extract 902 (H ₂ O)	Extract 903-a (70 per cent alcohol)	Extract 903 (H ₂ O)
Material extracted, <i>per cent.</i> ..	19.4	11.5	21.8	8.5	24.9	5.9
N per cc., <i>mg.</i>	0.22	0.14	0.25	0.12	0.35	0.09
Cu ₂ O per 10 cc., <i>gm.</i>	0.0116	0.0170	0.0142	0.0132	0.021	0.005
Activity.....	+	++++	++	+++	++	+++

* Each alcohol extraction was followed by a water extraction as stated in the text.

use of 80 per cent and 70 per cent alcohol instead of 85 per cent alcohol (Table VII).

In concentrations lower than 95 per cent, alcohol removed the active material, but 70 per cent alcohol removed practically all the water-soluble gum, Substance A, since water extracts following this treatment gave no higher reducing substances after hydrolysis than a constant factor given by successive inactive extracts of pollen residues (see page 575). The water extract, Extract 903, was active in all the cases tested.

50 gm. of absolute and 95 per cent alcohol-washed pollen were treated with six changes of 70 per cent alcohol in portions of 125 cc., with frequent shaking. A 5 gm. sample of the pollen was removed and further washed with 70 per cent alcohol to remove

the last traces of soluble material. 3 gm. of this were extracted with 100 cc. of bicarbonate fluid giving Extract 871, Substance B.

The remainder of the pollen was extracted with 100 cc. of water and the clear filtrate precipitated with an equal amount of alcohol, giving a flocculent precipitate, Precipitate 873 of 0.3 per cent. This precipitate was removed and the clear solution was precipitated by addition of alcohol to 90 per cent concentration. The resulting material, Precipitate 874 of 1.3 per cent, was similar in character to Precipitate 876 and was Substance A not completely removed by the 70 per cent alcohol from such a large amount of pollen.

In order to have an extract of ragweed pollen completely free from Substance A for desensitization tests (see page 580) a sample

TABLE VIII
Analysis of Extracts of Pollen Residues

3 gm. per 100 cc. of saline were used.

Pollen residues	N per cc.	Cu ₂ O in 10 cc.	Substance	Activity
	<i>mg.</i>	<i>gm.</i>		
Pollen after absolute and 95 per cent alcohol extraction.....	0.26	0.0353	A + B	++++
Pollen Extract 871 after 70 per cent alcohol extraction.....	0.11	0.0164	B + A	+++
Pollen Extract 883 after 50 per cent alcohol extraction.....	0.11	0.0090	B	+++

of the absolute and 95 per cent alcohol-washed pollen after exhaustive extraction with 70 per cent alcohol was finally extracted with two 100 cc. volumes of 50 per cent alcohol. The dry pollen residue was extracted with 3 gm. to 100 cc. of physiologic salt, and gave Extract 883, which analysis showed was free from Substance A (Table VIII).

Extract 883, Substance B, in desensitization tests, completely desensitized sites so that routine ragweed extracts gave no reaction. This showed that Substance B contained the biologically active material of the ragweed pollen, indicating there was only one active material in ragweed extracts.

Solutions Made from 0.2 Gm. of Precipitates in 100 Cc. Water

Precipitate No.	Pollen	Cu ₂ O per 10 cc.	Substance	Activity
	<i>per cent</i>	<i>gm.</i>		
873	0.3	0.0083	B	+++
874	1.3	0.0190	A + B	+
876	4.6	0.0268	A + B	+

Precipitate 873 resulting at 50 per cent alcoholic concentration from a water extract of 70 per cent alcohol-washed pollen was active in all cases tested. This was Substance B. Precipitates 874 and 876, inactive in about 10 per cent of the cases tested, were undoubtedly carbohydrate material containing small amounts of Substance B.

B. Isolation and Identification of Substance B

Substance B, Extract 883, gave positive biuret reaction and precipitation with Millon's reagent, phosphotungstic acid, and tannic acid. It contained nitrogen and its solubility in distilled water indicated protein material of albuminous nature.

45 gm. of ragweed pollen were washed with absolute and 95 per cent alcohol and finally with about 600 cc. of 85 per cent alcohol until most of the brownish gum had been removed. This facilitated the purification of the small amount of protein. The dry pollen residue was extracted with 150 cc. of water. The filtered water extract was treated with 150 cc. of saturated ammonium sulfate solution. After standing overnight no precipitate resulted and globulins were considered to be absent. The solution was saturated with ammonium sulfate and an albuminous substance separated, floating on the surface. The precipitate was dissolved in 100 cc. of water and 60 gm. of ammonium sulfate were added, when a permanent turbidity resulted. The pH was adjusted to 4.6 with 0.2 N sulfuric acid. An albuminous material, yellow in color, separated and after standing for 4 days it had a granular appearance and settled to the bottom of the flask. This precipitate was dissolved in water and similarly precipitated five times. The ammonium sulfate filtrate was practically inactive. The final precipitate was a very faint yellow in color and typically albuminous in nature. After being dissolved in water and dialyzed free from sulfate in a hardened membrane, the final solution, Extract

973, after Seitz filtration, measured 50 cc. It contained 0.02 mg. of nitrogen per cc., gave a positive biuret test, but negative Molisch test, showing the absence of carbohydrate material. On evaporation 10 cc. gave 0.0025 gm. of material.

The biological tests for comparing this preparation with routine ragweed extracts were carried out according to the method shown on page 570. Extract 973 desensitized passive sites completely so that subsequent tests with routine extracts were negative. This proved to be the case in all experiments done and showed that this preparation contained all of the active substances of ragweed pollen.

A 1:1000 dilution of Extract 973 containing 0.0000002 gm. of material per cc. and 0.00000002 gm. of nitrogen per cc. gave a positive reaction in a moderately sensitive case. Therefore we do not feel that the failure to demonstrate differences in nitrogen content by a Kjeldahl determination on active extracts after tryptic digestion (Coca and Grove (7) and Black and Moore (8)) was sufficient evidence on which to conclude that protein was absent and was not the active material.

Active material was precipitated by saturation with magnesium sulfate, but precipitation was not complete as subsequent saturation with ammonium sulfate resulted in further precipitation of active material.

188 gm. of ragweed pollen were washed with absolute and 95 per cent alcohol and finally 85 per cent alcohol as described. The dried residue was extracted for 5 hours with distilled water and filtered. The filtrate and washings, measuring 600 cc., were saturated with ammonium sulfate, and the precipitate filtered off. A second water extraction of the pollen residue on saturation with ammonium sulfate gave only a small amount of precipitate, showing that one extraction with water removed most of the active material. The two precipitates were combined, washed well with saturated ammonium sulfate solution, dissolved in water, and dialyzed in a hardened membrane against distilled water until free from sulfate. The solution was filtered and precipitated five times by about three-fourths saturation with ammonium sulfate and the pH was adjusted to 4.6. After the last precipitation, the precipitate was practically colorless. It was dissolved in water, dialyzed free from sulfate, and after Seitz filtration measured 200 cc. This clear solu-

tion, Extract 991, gave a froth on shaking, contained 0.2 mg. of nitrogen per cc., gave a positive biuret reaction, a precipitate with picric acid and with Millon's reagent, but a negative Molisch test. It was levorotatory. On evaporation at 100° a coagulated albuminous material precipitated, amounting to 0.0009 gm. per cc. It contained no phosphorus and 10 cc. of the solution after hydrolysis and clearing with mercuric nitrate gave no reduction. With this preparation it was impossible to demonstrate a precipitin test with ragweed-sensitive serum. The desensitization tests with this preparation are shown on page 571. By similar tests it was shown to be identical with Extract 973.

The remainder of the solution, about 160 cc., was precipitated with ammonium sulfate at pH 4.6. The precipitate was dissolved in 15 cc. of water and dialyzed free from SO_4 against distilled water. The final volume, 30 cc., was evaporated to 0.5 volume in a vacuum desiccator, when it was treated with 190 cc. of absolute alcohol. A white flocculent precipitate separated which was removed, dried at 100°, and submitted to analysis. Evaporation of the alcohol showed complete precipitation. The dried material produced violent sneezing in a slightly sensitive case.

Analysis of material dried at 100°: N 12.59, C 47.90, H 6.09, residue 4.90.

Percentage calculated after correcting for the 4.90 per cent residue: N 13.52, C 50.37, H 6.40.

The residue was composed of a small amount of Ca and SO_4 . Since the material was precipitated from a solution containing sulfuric acid, a small amount would be held by the protein and not removed completely by dialysis. No phosphorus was present.

The typical elementary analysis, high nitrogen content, as well as a negative Molisch test, no reduction after hydrolysis, and the absence of phosphorus showed the material was a simple protein. The material was characteristic of albumin since it was soluble in distilled water, precipitated by saturation with ammonium sulfate, coagulated on boiling with resulting lowering of activity and solubility, and gave protein tests.

Although very active in high dilutions and giving direct skin tests as great or greater than routine extracts, when dilutions were made on a nitrogen basis, the purified material probably became

somewhat less active during the process of isolation. In desensitization tests the purified material, on a nitrogen basis, desensitized sites against routine extracts, on a nitrogen basis. The purified material was not as strong as might have been expected since about two-thirds of the nitrogen in routine ragweed extracts has been shown to be inactive.

The elementary analyses on the small amounts of material obtainable were made possible by the use of the microchemical methods of Pregl through the courtesy of Professor Niederl, Mr. Silbert, and Mr. Saschek of New York University, to whom we wish to express our thanks and appreciation.

SUMMARY

1. The direct skin test while valuable for establishing activity was not satisfactory for determining the relative amount of active substance in various solutions. Such accurate determinations were made possible by quantitative desensitization of passively sensitized sites.

2. Cold absolute and 95 per cent alcohol removed water-soluble reducing substances, nitrogenous material, and acidity, but no active substance. 70 per cent alcohol removed a large amount of inert gum and some active substance leaving the bulk of active substance in the pollen residue.

3. From the pollen of the giant ragweed the active substance that produces the symptoms and skin reactions in individuals clinically sensitive to ragweed pollen (hay fever) has been isolated. This substance by chemical reactions and elementary analysis was typical of a simple protein of albuminous nature. Desensitization experiments have shown that this was the only active substance.

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